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LATHAM & WATKINS LLP

January 22, 2013

VIA EMAIL

P65Public.Comments@oehha.ca.gov

Ms. Cynthia Oshita
Office of Environmental Health Hazard Assessment
1001 I Street
Sacramento, California 95814

Dear Ms. Oshita:

On behalf of Ferro Corporation, we are submitting the enclosed information for consideration by OEHHA as it prepares cancer hazard identification materials for butyl benzyl phthalate (BBP) (CASRN 85-68-7). Ferro is a major producer of BBP.

There is a robust set of data for BBP. For the reasons given in our September 20, 2011 comments regarding prioritization, as well as the additional information herein, Ferro believes the evidence does not support listing of BBP under Proposition 65 as a carcinogen. NTP, which conducted the bioassays on BBP, has not considered BBP for listing in the Report on Carcinogens. IARC and the EU have not classified BBP as a possible or probable carcinogen. There are no human carcinogenicity data for BBP. The great weight of evidence is that BBP is not genotoxic. Tumor formation has been inconsistent and marginal in cancer bioassays, and other animal data do not support a concern for carcinogenicity. Further, biomonitoring data demonstrate that human exposure levels for BBP are extremely low.

If OEHHA has any questions or wishes additional information, please contact the undersigned at the telephone or email given above..

Sincerely,

Ann Claassen
of LATHAM & WATKINS LLP

Enclosure

cc: Alan Olson, Ferro Corporation

Submission of

Ferro Corporation

on

SCIENTIFIC EVIDENCE PERTAINING TO BUTYL BENZYL PHTHALATE (BBP)

for consideration by the

State of California
Office of Environmental Health Hazard Assessment

for Preparation of

Hazard Identification Materials under Proposition 65

January 22, 2013

TABLE OF CONTENTS

INTRODUCTION

Ferro Corporation (Ferro) is submitting the information herein to the California Office of Environmental Health Hazard Assessment (OEHHA) regarding scientific evidence pertaining to preparation of hazard identification materials for butyl benzyl phthalate (BBP) (CASRN 85-68-7).[1] Ferro is a major producer of BBP.

OEHHA's preliminary toxicological review describes three long-term cancer bioassays and two short-term carcinogenicity studies conducted on BBP, all conducted by the National Toxicology Program.[2] The review also identified *in vivo* and *in vitro* genotoxicity data and mechanistic studies, including estrogenic activity assays. For prioritization of BBP by the Carcinogenic Identification Committee,[3] Ferro provided comments examining the significance of these studies for evaluating the potential carcinogenicity of BBP, plus other relevant information. A copy of those comments is provided as Attachment 1 to these comments.[4] This present document provides additional information.

In early January, 2013, we conducted a literature search for Butyl Benzyl Phthalate (CAS RN 85-68-7) on the National Center for Biomedical Information (NCBI) database using the PubMed search tools. Our search identified 129 titles dating back to 1980. Most of these relate to ecotoxicity or developmental toxicity. We selected 18 articles regarding higher-order mammalian toxicity studies relevant to assessing carcinogenic potential; these are listed in Appendix A. Only the first two have been published since our prior submission.

Our search found a bioassay that was not discussed in OEHHA's preliminary assessment or our prior submission (Kohno et al., 2004); it is discussed below. Nearly all other titles in Appendix A describe *in vitro* studies which examined genomic or endocrine disruption markers, which, as discussed below, are of limited usefulness for predicting the carcinogenicity of BBP.

Ferro believes that, in agreement with the implied or explicit assessments of NTP, IARC, Canada, and the European Union,[5] the evidence is insufficient to list BBP as "known to the State of California to cause cancer."

The following describes first the uses of BBP, then discusses the epidemiological, toxicological, genotoxicity and mechanistic data, and then discusses exposure data.

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1 *See* OEHHA, Announcement of Chemical Selected by OEHHA for Consideration for Listing by the Carcinogen Identification Committee and Request for Relevant Information on the Carcinogenic Hazards of Butyl Benzyl Phthalate (Nov. 26, 2012), *available at* http://www.oehha.ca.gov/prop65/CRNR_notices/state_listing/data_callin/note112312.html.

2 OEHHA, Butyl Benzyl Phthalate (undated), available at http://www.oehha.ca.gov/prop65/public_meetings/CIC101211/101211ButBenzPhthalate_CIC.pdf.

3 *See* OEHHA, Prioritization: Chemicals for Consultation by the Carcinogen Identification Committee (July 22, 2011 Notice), available at http://www.oehha.org/prop65/public_meetings/prior072211.html; OEHHA, October 12 and 13, 2011 Meeting of the Carcinogen Identification Committee (Sept. 30, 2011; posted Sept. 23, 2011), *available at* http://www.oehha.org/prop65/public_meetings/cic092311.html; OEHHA, Meeting Agenda and presentations of the October 12, 2011 Carcinogen Identification Committee (Oct. 13, 2011), *available at* http://www.oehha.org/prop65/public_meetings/cic101211.html; OEHHA, Meeting Synopsis and Slide Presentations Carcinogen Identification Committee Meeting Held on October 12, 2011 (Nov. 02, 2011), *available at* http://www.oehha.org/prop65/public_meetings/cic101211synop.html.

4 Some typographic errors that were in the 2011 submission are corrected in this version.

5 NTP (2011); IARC (1982; 1999); IPCS (1999); ECB (2007). *See also* Section IV of Attachment 1.

I. Uses of BBP

The primary use of BBP in the US is as a plasticizer in vinyl flooring (sheet and tile), carpet tile, and other building materials such as wallpaper and weather stripping. It is also used in caulks, adhesives, artificial leather, tarps, and automotive trim.

A number of reviews of BBP and articles in the literature list two types of uses which are no longer applicable – toys and cosmetics.

- Prior to this century, there was some limited use of BBP in toys; however, BBP has been prohibited in toys and child care articles since 1999 in the European Union (EU)[6] and since 2009 in the United States.[7] The EU prohibition had led to deselection of BBP in toys even before the ban in the US.

- The US Cosmetic Ingredient Review Expert Panel reviewed BBP in 1992 and found it safe for use in cosmetics (CIR, 1992), and there has been some limited use of BBP in cosmetics. However, BBP is now banned from cosmetics in the EU[8] and for many years has been used minimally, if at all, in cosmetics in the US.

If OEHHA has any specific questions regarding uses of BBP in the United States, Ferro would be pleased to provide answers to the best of its ability.

II. Epidemiology Data

There are no human studies on BBP carcinogenicity.

III. Animal Carcinogenicity Bioassays

A. Long-term Bioassays

As discussed in our prior submission (Section I of Attachment 1), NTP has examined the carcinogenicity of BBP in three rodent bioassays employing two species: rats and mice (NTP, 1982; 1997a; 1997b). The results are briefly summarized in Table 1; see also Appendix A of Attachment 1. There were no tumors observed in mice. Comparison of the results in rats from these three assays reveals a lack of consistency in tumor sites among the studies, even when tested in the same strain, so that the weight of evidence for a given tumor type in rats is at most equivocal.

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[6] A temporary ban in 1999 was made permanent in 2005. Directive 2005/84/EC of the European Parliament and of the Council of 14 December 2005, amending for the 22nd time Council Directive 76/769/EEC on the approximation of the laws, regulations and administrative provisions of the Member States relating to restrictions on the marketing and use of certain dangerous substances and preparations (phthalates in toys and childcare articles), O.J. L344:40 (Dec. 27, 2005).

[7] Consumer Product Safety Improvement Act of 2008, Section 108(a), P.L. 110-314 (Aug. 14, 2008).

[8] Council Directive of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products (76/768/EEC), Annex II (consolidated version), *available at* http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:1976L0768:20100301:en:PDF.

Table 1. Cancer Bioassay Results

Lesion	Male Mice	Female Mice	Male Rats	Female Rats
MNCL	**No**	**No**	**No** NTP 97a – No NTP 97b – w/in historical range	**Equivocal** NTP 82 – Yes NTP 97a – No NTP 97b – w/in historical range
Pancreatic Tumor	**No**	**No**	**Equivocal** NTP 97a – Yes NTP 97b -- Diet link; No tumor ↑ in weight-controlled animals	**No**
Bladder	**No**	**No**	**No**	**No** NTP 82 – No NTP 97a & 97b, – Not statistically significant
Mammary	**No**	**No**	**No** NTP 97b – reduced	**No** NTP 97b – reduced
Tumor Promotion	**N/A**	**N/A**	**No** – negative in all 3 studies	**No** – negative in all 3 studies

B. Short-term Bioassays

Two short-term bioassays were discussed in our previous submission (Section I.D of Attachment 1). In a short-term intraperitoneal injection study in mice, there was no increase in pulmonary tumors (Theiss, et al., 1977). In a short-term co-carcinogenicity study in rats, BBP administration by gavage *inhibited* tumor formation by dimethylbenz[a]anthracene (DMBA), and reduced mammary DMBA-DNA adduct formation (Singletary, et al., 1997).

We have identified another study that used a standard initiation-promotion tumor model (Kohno, et al., 2004). Forty weeks of treatment with BBP did not promote dimethyl aminobiphenyl prostate tumor formation and also did not induce tumor formation.

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Thus, *in vivo* animal bioassays indicate that BBP has low potential to cause cancer in humans.

IV. Genotoxicity Testing

As discussed in our prior comments (Section II of Attachment 1), BBP has been tested in a variety of *in vitro* and *in vivo* genetic toxicity assays for genetic toxicity endpoints and for the

ability to induce morphologic transformation. The *in vitro* assays were conducted with bacterial, yeast and mammalian cell systems. *In vivo* assays were conducted in mice, rats and *Drosophila*. In most assays, BBP was negative; in the remainder, results were equivocal.

The results of the BBP genotoxicity assays are summarized in Table 2 below and in Appendix B (Tables 1-4) of Attachment 1. As the table shows, the great weight of the evidence indicates that BBP is not genotoxic.

Table 2. Genotoxicity Assay Results

Type	Positive	Negative	Equivocal
In vitro mutation, prokariotic cells	**0**	**6**	**0**
In vitro mutation, mammalian cells	**0**	**2**	**1**
In vitro chromosome, cell morphology, mammalian	**0**	**3**	**1**
In vivo	**1**	**3**	**1**
TOTAL	**1**	**14**	**3**

V. Mechanistic Data

A. Estrogenicity

In its preliminary toxicology review, OEHHA cited data concerning estrogenic activity. Appendix C of our prior submission (Attachment 1) was an opinion provided by Dr. Timothy R. Zacharewski of Michigan State University. Dr. Zacharewski explained that the weight of the evidence is that BBP is not estrogenic. In particular, it is negative for estrogenicity in the definitive *in vivo* studies. Dr. Zacharewski's opinion explains that *in vitro* data are not useful for evaluating the carcinogenicity of BBP, stating:

> Although *in vitro* assays can be useful to identify chemicals that interact with the estrogen receptor and to elucidate mechanisms of action, they do not replicate *in vivo* conditions. Overall, *in vitro* assays have a poor record of predicting *in vivo* responses, especially for complex diseases such as breast cancer. Consequently, it is my opinion that *in vitro* assays are not useful for evaluating the potential carcinogenicity of BBP.

Dr. Zacharewski concluded that the weight of evidence indicates that BBP is not carcinogenic via an estrogenic mode.

Dr. Zacharewski has recently completed a sabbatical at the US Environmental Protection Agency (USEPA) in North Carolina, where he evaluated the use of data from High Throughput Screening Assays and genomics-based assays for predicting toxicological outcomes. He was specifically tasked with identifying challenges likely to be encountered with the use of *in vitro* assays in risk assessment. Dr. Zacharewski has prepared a manuscript presenting his findings. The manuscript is currently in USEPA review; clearance by USEPA is anticipated soon. We will provide the manuscript to OEHHA as soon as it is available for distribution.

B. Other Mechanistic Data

Our prior submission discussed two other publications OEHHA identified as "mechanistic considerations" and explained that the studies do not provide substantial support for a concern of potential carcinogenicity of BBP in humans. See Section III.C of Attachment 1.

C. Metabolism

In most mammalian species the primary metabolites of BBP are excreted in the urine as unconjugated monobutyl and monobenzyl esters. Examination of urinary metabolites of rats following oral administration of 3.6 mmol BBP/kg/d for 3 days indicated that approximately 70% of the metabolites were unconjugated monoesters, while the remainder was conjugated (Eigenberg, et al., 1986). Eigenberg, et al. also showed that urinary metabolites of BBP account for about 50% of a range of oral doses to F-344 rats. Nativelle, et al. (1999) showed essentially the same in female Wistar rats. In dogs, however, only about 10% of an oral dose was metabolized (Erikson, 1965). Each of these studies also produced evidence of changes in phthalate metabolism occurring with increasing oral dose. The half-life of BBP in blood of rats is 10 minutes, while the blood half-life of monoester metabolites of BBP is 5.9 h (Eigenberg, et al. 1986).

The predominant monester differs with mammalian species. The rat preferentially hydrolyzes BBP to form the monobutyl ester (Eigenberg, et al., 1986; Mikuriya, et al., 1988; Monsanto, 1996a; Monsanto, 1996b. In the rat BBP yields approximately 16% MBzP and 44% MBuP on a molar basis (Eigenberg, et al. 1986).

Anderson, et al. (2001) exposed human volunteers to low doses of isotope-labeled BBP and measured the metabolites in urine. A single oral dose of 253 or 506 µg of BBP was administered and 24-hour urine samples were collected for analysis. On average, 140 µg and 323 µg of monobenzyl phthalate (MBzP) and 20 µg of monobutyl phthalate (MBuP) was eliminated. The MBuP was measureable in the high exposure group only. Anderson's data show that in humans, in contrast to the rat, BBP is preferentially hydrolyzed to form MBzP (73% on a molar basis) over MBuP (6% on a molar basis).

VI. Human Exposure

In our prior comments (Section V of Attachment 1), we discussed the biomonitoring data published by the Centers for Disease Control and Prevention (CDC)[9] and showed that the lowest BBP dose levels in rats that produce (equivocal) evidence of tumors are 250,000 to 4,000,000-fold above the levels for adult human exposure and 73,000 to 1,100,000-fold above the levels for children's exposure.

Other sources of exposure data support these findings. Guo and Kannan (2011) measured concentrations of BBP in indoor dust in China and the US (Albany NY) and calculated estimates of daily exposure via dust ingestion and dermal dust exposure. For the US age-group estimated to have the highest exposure (toddlers), exposure from ingestion of dust was estimated to be 0.1 micrograms BBP per kilogram per day (ug/kg/day) and from dust dermal absorption was 0.002 ug/kg/day. Wittassek, et al. (2010) back-calculated BBP exposures from US biomonitoring studies of 0.73-0.5 ug/kg/day at the median and 2.5-3.3 ug/kg/day at the 95th percentile. USEPA (2005) estimated exposures to BBP, among other chemicals, via ingestion (including dust ingestion) and inhalation. The estimated median potential exposure for preschool children was 10.0 ug/kg/day and the potential absorbed dose was estimated to be 0.29 ug/kg/day.[10] These values align well with those derived from the CDC biomonitoring, showing geometric mean aggregate exposure to BBP to be 1.1 ug/kg/day for children aged 6-11.[11]

At a January 13, 2013 meeting of OEHHA and Ferro representatives, OEHHA asked whether there is biomonitoring data using metabolites other than the monoester. We assume this question follows from the finding for some other phthalates that metabolites other than the monoester are more readily detected than is the monoester. We are not aware of biomonitoring for other BBP metabolites. However, we would note that, while another metabolite might effectively lower the detection limit, and thus increase the percentage of the population showing detectable BBP metabolite, the relative ratios of metabolites to the BBP dose will remain the same. Thus, the median and 95th percentile values derived from other metabolites will be similar to those derived from the monoester.

CONCLUSION

Ferro believes that the totality of data for BBP demonstrates that concern for BBP induced carcinogenicity is very low. The evidence is not sufficient to list BBP as a human carcinogen.

Ferro would be pleased upon request to provide, to the extent possible, additional information to assist OEHHA in its preparation of Hazard Identification Materials.

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[9] The prior submission cited CDC (2011). Updated tables are available as CDC (2012), but the values for BBP are the same as in the 2011 tables.

[10] These are the values for children observed in Ohio. Levels for children in North Carolina were slightly lower.

[11] See Section V of Attachment 1.

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CDC (2012). Urinary mono-benzyl phthalate (MBzP) (creatinine corrected), in Fourth National Report on Human Exposure to Environmental Chemicals, Updated Tables, February 2012, available at http://www.cdc.gov/exposurereport/pdf/FourthReport_UpdatedTables_Sep2012.pdf.

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Erickson, N. G., et al., (1965). The metabolism of diphenyl phthalate and butylbenzyl phthalate in the beagle dog. Dissert. Abs. 26(5), 3014–3015.

Guo, Y. and Kannan, K. (2011). Comparative Assessment of Human Exposure to Phthalate Esters from house Dust in China and the United States. Environ. Sci. Tech. 45:3788-3794 (2011).

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Mikuriya, H., Ikemoto, I. and Tanaka, A. (1988). Urinary metabolites contributing to testicular damage induced by butylbenzyl phthalate. Jikeikai Med. 35, 403-409.

Monsanto (1996a). Project No. xx-96-247. Study to evaluate the effect of monobutyl phthalate on uterine growth in immature female rats after oral administration. CT:/R/1279.

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NTP (1997a). Toxicology and carcinogenesis studies of butyl benzyl phthalate in F344/N rats (feed studies). National Toxicology Program, Technical Report No. 458, NIH publication No. 97-3374.

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APPENDIX A

Pertinent Articles on BBP Identified in PubMed Search

1. Benzyl butyl phthalate induces necrosis by AhR mediation of CYP1B1 expression in human granulosa cells.

   Chen HS, Chiang PH, Wang YC, Kao MC, Shieh TH, Tsai CF, Tsai EM.

   Reprod Toxicol. 2012 Jan;33(1):67-75. doi: 10.1016/j.reprotox.2011.11.004. Epub 2011 Nov 25.

   PMID: 22138065 [PubMed - indexed for MEDLINE]

2. Xenoestrogens down-regulate aryl-hydrocarbon receptor nuclear translocator 2 mRNA expression in human breast cancer cells via an estrogen receptor alpha-dependent mechanism.

   Qin XY, Zaha H, Nagano R, Yoshinaga J, Yonemoto J, Sone H.

   Toxicol Lett. 2011 Oct 10;206(2):152-7. doi: 10.1016/j.toxlet.2011.07.007. Epub 2011 Jul 12.

   PMID: 21771643 [PubMed - indexed for MEDLINE]

3. In utero exposure to butyl benzyl phthalate induces modifications in the morphology and the gene expression profile of the mammary gland: an experimental study in rats.

   Moral R, Santucci-Pereira J, Wang R, Russo IH, Lamartiniere CA, Russo J.

   Environ Health. 2011 Jan 17;10(1):5. doi: 10.1186/1476-069X-10-5.

   PMID: 21241498 [PubMed - indexed for MEDLINE] **Free PMC Article**

4. Proteomic analysis of proteins secreted by HepG2 cells treated with butyl benzyl phthalate.

   Choi S, Park SY, Kwak D, Phark S, Lee M, Lim JY, Jung WW, Sul D.

   J Toxicol Environ Health A. 2010;73(21-22):1570-85. doi: 10.1080/15287394.2010.511583.

   PMID: 20954082 [PubMed - indexed for MEDLINE]

5. Butyl benzyl phthalate suppresses the ATP-induced cell proliferation in human osteosarcoma HOS cells.

   Liu PS, Chen CY.

   Toxicol Appl Pharmacol. 2010 May 1;244(3):308-14. doi: 10.1016/j.taap.2010.01.007. Epub 2010 Jan 28.

   PMID: 20114058 [PubMed - indexed for MEDLINE]

6. Estrogen and xenoestrogens in breast cancer.
Fernandez SV, Russo J.
Toxicol Pathol. 2010 Jan;38(1):110-22. doi: 10.1177/0192623309354108. Epub 2009 Nov 21. Review.
PMID: 19933552 [PubMed - indexed for MEDLINE] **Free PMC Article**

7. The role of developmental toxicity studies in acute exposure assessments: analysis of single-day vs. multiple-day exposure regimens.
Davis A, Gift JS, Woodall GM, Narotsky MG, Foureman GL.
Regul Toxicol Pharmacol. 2009 Jul;54(2):134-42. doi: 10.1016/j.yrtph.2009.03.006. Epub 2009 Mar 21.
PMID: 19306903 [PubMed - indexed for MEDLINE]

8. Butyl benzyl phthalate: effects on immune responses to ovalbumin in mice.
Dearman RJ, Betts CJ, Beresford L, Bailey L, Caddick HT, Kimber I.
J Appl Toxicol. 2009 Mar;29(2):118-25. doi: 10.1002/jat.1388.
PMID: 18816477 [PubMed - indexed for MEDLINE]

9. [Effects of butyl benzyl phthalate on neurobehavioral development of rats].
Zhuang MZ, Li YF, Li T.
Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi. 2008 May;26(5):285-8. Chinese.
PMID: 18727871 [PubMed - indexed for MEDLINE]

10. The plasticizer butyl benzyl phthalate induces genomic changes in rat mammary gland after neonatal/prepubertal exposure.
Moral R, Wang R, Russo IH, Mailo DA, Lamartiniere CA, Russo J.
BMC Genomics. 2007 Dec 6;8:453.
PMID: 18062813 [PubMed - indexed for MEDLINE] **Free PMC Article**

11. DNA methylation of estrogen receptor alpha gene by phthalates.
Kang SC, Lee BM.
J Toxicol Environ Health A. 2005 Dec 10;68(23-24):1995-2003.
PMID: 16326419 [PubMed - indexed for MEDLINE]

12. Lack of modifying effects of 4-ter t-octylphenol and benzyl butyl phthalate on 3,2-dimethyl-4-aminobiphenyl-induced prostate carcinogenesis in rats.
Kohno H, Suzuki R, Sugie S, Tsuda H, Tanaka T.
Cancer Sci. 2004 Apr;95(4):300-5.

PMID: 15072586 [PubMed - indexed for MEDLINE]

13. Decreased anogenital distance and increased incidence of undescended testes in fetuses of rats given monobenzyl phthalate, a major metabolite of butyl benzyl phthalate.
Ema M, Miyawaki E, Hirose A, Kamata E.
Reprod Toxicol. 2003 Jul-Aug;17(4):407-12.
PMID: 12849851 [PubMed - indexed for MEDLINE]

14. Full activation of estrogen receptor alpha activation function-1 induces proliferation of breast cancer cells.
Fujita T, Kobayashi Y, Wada O, Tateishi Y, Kitada L, Yamamoto Y, Takashima H, Murayama A, Yano T, Baba T, Kato S, Kawabe Y, Yanagisawa J.
J Biol Chem. 2003 Jul 18;278(29):26704-14. Epub 2003 May 8.
PMID: 12738788 [PubMed - indexed for MEDLINE] **Free Article**

15. Response of MCF-7 human breast cancer cells to some binary mixtures of oestrogenic compounds in-vitro.
Suzuki T, Ide K, Ishida M.
J Pharm Pharmacol. 2001 Nov;53(11):1549-54.
PMID: 11732758 [PubMed - indexed for MEDLINE]

16. The plasticizer benzyl butyl phthalate (BBP) inhibits 7,12-dimethylbenz[a]anthracene (DMBA)-induced rat mammary DNA adduct formation and tumorigenesis.
Singletary K, MacDonald C, Wallig M.
Carcinogenesis. 1997 Aug;18(8):1669-73.
PMID: 9276647 [PubMed - indexed for MEDLINE] **Free Article**

17. A review of the subchronic toxicity of butyl benzyl phthalate.
Hammond BG, Levinskas GJ, Robinson EC, Johannsen FR.
Toxicol Ind Health. 1987 Jun;3(2):79-98.
PMID: 3617072 [PubMed - indexed for MEDLINE]

18. Distribution, excretion, and metabolism of butylbenzyl phthalate in the rat.
Eigenberg DA, Bozigian HP, Carter DE, Sipes IG.
J Toxicol Environ Health. 1986;17(4):445-56.
PMID: 3959124 [PubMed - indexed for MEDLINE]

ATTACHMENT 1

Ferro Submission in Advance of CIC Prioritization Meeting

September 20, 2011

Comments of

Ferro Corporation

on

SCIENTIFIC EVIDENCE PERTAINING TO BUTYL BENZYL PHTHALATE (BBP)

for consideration by the

State of California
Office of Environmental Health Hazard Assessment
Science Advisory Board Carcinogen Identification Committee

with respect to

Prioritization of Chemicals for Possible Preparation of Hazard Identification Materials under Proposition 65

September 20, 2011

Typographic errors corrected, January 22, 2013

EXECUTIVE SUMMARY

Ferro Corporation (Ferro) is submitting these comments to the California Office of Environmental Health Hazard Assessment (OEHHA) on the extent of the scientific evidence pertaining to the selection of butyl benzyl phthalate (BBP) (CASRN 85-68-7) for possible preparation of hazard identification materials. BBP is one of 39 chemicals to be discussed at the October 12-13, 2011 meeting of the Proposition 65 Carcinogen Identification Committee (CIC). These comments are submitted to assist the CIC in providing advice to OEHHA on the prioritization of BBP. Ferro is a major producer of BBP.

The evidence strongly supports a low prioritization for BBP. This is shown by review of the studies cited by OEHHA in its compilation of the preliminary toxicological review of BBP (summarized in Appendix A) and by other relevant information.

The body of these comments provides more detailed analysis of the BBP data. The complete database for BBP, including all studies completed by the National Toxicology Program (NTP), reveals that, at most, BBP has low potential to produce tumors in laboratory animals and does not produce rare tumors or induce tumors at an early onset. Findings from the BBP database include:

- the great weight of the evidence is that BBP is not genotoxic;
- no increase in tumors was observed in mice treated with BBP (NTP, 1982);
- a statistically significant increase in mononuclear cell leukemia (MNCL), a common tumor in F344 rats of questionable relevance to humans, was observed in female rats in one study (NTP, 1982), but this finding was not reproducible (NTP, 1997a);
- there was no other statistically significant increase in tumors in female rats;
- the marginal increase in pancreatic tumors in female rats in one study (NTP, 1997a) was not repeated in a subsequent 2-year bioassay (NTP, 1997b);
- there was a marginal increase in urinary bladder tumors (NTP, 1997a), but tumors were significantly increased in the subsequent bioassay only after 32 months (NTP,1997b);
- a statistically significant increase in pancreatic tumors was observed in male rats fed *ad libitum* (NTP, 1997a; NTP, 1997b); however, no such increase was seen in male rats kept on a weight-restricted diet for 2 years, indicating that diet may play an important role;
- in an 8-week intraperitoneal injection study (a standard tumor induction model), BBP caused no pulmonary tumors after 24 weeks (Theiss, et al., 1977); and
- in a short-term co-carcinogenicity study, BBP inhibited tumor formation by DMBA (Singletary, et al., 1997).

Thus, taken as a whole, the bioassay data for BBP do not reveal any strong tumor responses. This can reasonably be concluded from the lack of response in mice; inability to reproduce the leukemia response in female rats; absence of rare or early onset tumors; and the

low incidences and influence of the diet or length of study on the pancreatic tumors in male rats or the urinary bladder tumors in female rats.

The genotoxicity information on BBP, which includes a large number of *in vitro* and *in vivo* assays, is overwhelmingly negative. A summary of these studies is provided in Appendix B; a more detailed discussion is in the text.

OEHHA cites *in vitro* studies on estrogenicity and other mechanistic data. Timothy Zacharewski, Ph.D., a leading expert on *in vitro* studies and endocrine modulation studies, has reviewed the studies cited by OEHHA and provided an opinion, attached here as Appendix C, on the usefulness of that data for assessing BBP carcinogenicity. *In vitro* data have a poor record of predicting *in vivo* responses; in the definitive *in vivo* study, BBP is negative. Dr. Zacharewski concludes that the weight of evidence is that BBP is not estrogenic, and that *in vitro* estrogenicity studies are not useful for evaluating the potential carcinogenicity of BBP. The other mechanistic data cited by OEHHA do not provide a basis for concern for BBP carcinogenicity, because they involve a mechanism not relevant to humans, or are grounded in an erroneous interpretation of the data.

Tellingly, NTP, which conducted the three bioassays and many of the genotoxicity tests on BBP, has never formally considered BBP for listing in the Report on Carcinogens. Evaluations by other expert bodies have concluded that the potential for BBP to cause carcinogenicity in humans is, at most, marginal. The International Agency for Research on Cancer (IARC) has classified BBP as Group 3 (not classifiable as to its carcinogenicity to humans) (IARC, 1999). The International Programme on Chemical Safety, based on a 1998 assessment by Health Canada and Environment Canada, concluded that BBP can be considered, at most, possibly carcinogenic to humans (IPCS, 1999). OEHHA itself has previously reviewed the data for BBP and given it a low priority for consideration as a carcinogen (OEHHA, 1997). Most recently, the European Commission made a determination not to classify BBP as a carcinogen (ECB, 2007).

Biomonitoring data provide further reason to give a low priority to BBP. The substantial data provided by the Centers for Disease Control and Prevention (CDC) yields estimates of human exposure that, at the 95th percentile, are four to five orders of magnitude below the rat dose that produced equivocal evidence of tumors and, at the geometric mean, are six orders of magnitude below that dose. The CDC data also show that exposures to BBP are decreasing.

In summary, as has been concluded by several reviewing expert bodies, the animal data for carcinogenicity from BBP is at most marginal, and exposure to BBP is extremely low. Therefore, BBP should be given a low priority for preparation of hazard identification materials.

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INTRODUCTION

Ferro Corporation (Ferro) is submitting these comments to the California Office of Environmental Health Hazard Assessment (OEHHA) on the extent of the scientific evidence pertaining to the selection of butyl benzyl phthalate (BBP) (CASRN 85-68-7) for possible preparation of hazard identification materials. BBP is one of 39 chemicals to be discussed at the October 12-13, 2011 meeting of the Proposition 65 Carcinogen Identification Committee (CIC).[1] These comments are submitted to assist the CIC in providing advice to OEHHA on the prioritization of BBP. Ferro is a major producer of BBP.

OEHHA has applied the human and animal data screens of its prioritization process to BBP and other chemicals,[2] and then conducted a preliminary toxicological evaluation for each chemical that met the screening criteria.[3] No cancer epidemiology studies were identified for BBP. In the animal data screen, OEHHA identified chemicals for which any of the following criteria are met: two or more positive animal cancer bioassays; one positive animal cancer bioassay with findings of tumors at multiple sites or with malignant (or combined malignant and benign) tumors occurring to an unusual degree with regard to incidence, site, type of tumor or age at onset; or one positive animal cancer bioassay and evidence from a second animal cancer bioassay of benign tumors of a type known to progress to malignancy.[4] OEHHA indicates that a positive bioassay is one in which a statistically-significant increase in tumor formation occurs as a result of treatment with test material, or any increase in a biologically-significant tumor (rare tumor) is seen.[5]

The preliminary toxicological review describes three long-term cancer bioassays and two short-term carcinogenicity studies conducted on BBP. The cancer bioassays were all conducted by the National Toxicology Program. The review also identified *in vivo* and *in vitro* genotoxicity data and mechanistic studies, including estrogenic activity assays.

These comments examine the significance of these studies for evaluating the potential carcinogenicity of BBP, plus other relevant information. Review of the database demonstrates that concern for BBP-induced carcinogenicity is low and that BBP should be given a low priority for preparation of hazard identification materials.

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1 OEHHA, Prioritization: Chemicals for Consultation by the Carcinogen Identification Committee (July 22, 2011 Notice), available at http://www.oehha.org/prop65/public_meetings/prior072211.html.

2 OEHHA, Process for Prioritizing Chemicals for Consideration under Proposition 65 by the "State's Qualified Experts." (December 2004), available at http://www.oehha.ca.gov/prop65/CRNR_notices/state_listing/pdf/finalPriordoc.pdf.

3 A compilation of studies OEHHA identified as relevant during the preliminary toxicological evaluation for BBP are provided in OEHHA, Butyl Benzyl Phthalate (undated), available at http://www.oehha.ca.gov/prop65/public_meetings/CIC101211/101211ButBenzPhthalate_CIC.pdf.

4 OEHHA, Prioritization: Chemicals Identified for Consultation with the Carcinogen Identification Committee (July 2011), Reproductive and Cancer Hazard Assessment Branch, p. 3, available at http://www.oehha.ca.gov/prop65/public_meetings/CIC101211/101211ChemPriorCIC2011.pdf.

5 *Id.*

I. Animal Bioassay Evidence of BBP Carcinogenicity Is Limited or Negative; therefore BBP Should Be Given a Low Priority

There are no human studies on BBP carcinogenicity. NTP has examined the carcinogenicity of BBP in three rodent bioassays employing two species: rats and mice (NTP, 1982; 1997a; 1997b). There were no tumors observed in mice. Comparison of the results in rats from these three assays reveals a lack of consistency in tumor sites among the studies. At most, these studies indicate that BBP has a low potential to produce tumors in laboratory rats, as observations of tumors are either not repeated, or are dependent on dietary status or length of study and limited to a single species. In a short-term intraperitoneal injection study in mice, there was no increase in pulmonary tumors (Theiss, et al., 1977). In a short-term co-carcinogenicity study in rats, BBP administration by gavage *inhibited* tumor formation by dimethylbenz[a]anthracene (DMBA), and reduced mammary DMBA-DNA adduct formation (Singletary, et al., 1997). Thus, the evidence is that BBP has low potential to cause cancer in humans and it accordingly should be given a low priority for development of hazard identification materials.

The results of these animal carcinogenicity studies are briefly summarized in Appendix A. More detailed discussion follows here.

A. NTP, 1982: No tumor response in mice; an increase of MNCL in female rats of questionable significance

NTP first examined the carcinogenicity of BBP in rats and mice in 1982 (NTP, 1982). Groups of 50 male or female rats and fifty male or female mice were exposed to BBP via the diet, at levels of 0, 6000 or 12000 ppm (0, 300 and 600 mg/kg/day, rats and 0, 780, or 1560 mg/kg/day, mice). Male and female mice and female rats were exposed for 103 weeks. Due to poor survival, all male rats were sacrificed at weeks 29-30; this part of the study was later repeated (NTP, 1997a).

No treatment-related neoplasms were observed in mice. Survival was not affected. A dose dependent reduction in body weight in both sexes was the only treatment related effect in mice observed in this study. Further, non-neoplastic changes were all within the normal limits of incidence for B6C3F1 mice. The NTP concluded that, under the conditions of the bioassay, BBP was not carcinogenic for B6C3F1 mice of either sex.

As male rats were terminated early, only female rats were evaluated at study completion. The incidence of mononuclear cell leukemia (MNCL) in control, low and high dose animals was 7/49, 7/49 and 18/50, respectively. The increased incidence in the high dose group was significant ($p=0.011$) by pair-wise comparison and a trend analysis was significant as well ($p=0.006$). No other treatment related findings were observed. The incidence in the high dose group and the overall trend remained significant ($p=0.008$ and $p=0.019$, respectively) when compared with historical control data at the time. The NTP concluded that BBP was "probably carcinogenic for female F344/N rats, causing an increased incidence of mononuclear cell leukemias" (NTP, 1982).

However, in a separate publication, the authors of the NTP report discussed the significance of the MNCL observed from this study. The authors concluded, "Although of statistical significance, the increased incidence of myelomonocytic leukemia in the female rats receiving the high dose of BBP was considered to be of equivocal biological significance due to the considerable variation in the background incidence of myelomonocytic leukemia in Fischer 344 rats" (Kluwe, et al., 1982). This view was amplified in Caldwell (1999), which points out that MNCL is a common neoplasm in F-344 rats that occurs at a high but variable rate,[6] that MNCL is uncommon or does not occur in other mammalian species, and that it is a lesion for which there is no human correlate neoplastic lesion. Thomas, et al. (2007) assert there is a human correlate, but note that the mechanism for development of the tumor may be different between species. Thomas, et al. advocate a weight of the evidence approach to assessing MNCL observations.

Caldwell (1999) notes that repeated chronic studies of BBP by the NTP have failed to produce consistent findings of an increased incidence of MNCL in F-344 rats. Caldwell provides other examples of inconsistency in the incidence of MNCL in F-344 rats (in repeat studies) and concludes that MNCL in the F-344 rat, alone, is not a useful basis for determining that a substance presents a carcinogenic hazard to humans.

Furthermore, as discussed below, the finding of increased MNCL in female rats was not reproducible in a second bioassay. Therefore, the weight of the evidence is that the MNCL observations in the 1982 study cannot be considered indicative of potential carcinogenicity for BBP.

B. NTP, 1997a: Increase in MNCL not repeatable; low incidence of increased pancreatic tumors in male rats possibly related to diet

NTP conducted a second bioassay on BBP (NTP, 1997a). This study was conducted in groups of fifty male and female Fischer 344 rats. Rats were fed diets containing 3000, 6000, and 12000 ppm BBP for male rats (delivering approximately 0, 120, 240 or 500 mg/kg/day) and 6000, 12000, and 24000 ppm BBP for female rats (approximately 0, 300, 600 or 1200 mg/kg/day). In addition to a terminal sacrifice at 103 weeks, the protocol included periodic hematological evaluation and hormonal assays, and a 15-month interim sacrifice.

There were no differences in survival between exposed groups and their control. The mean body weight of high-dose male rats was 10% lower than control males, indicating that the maximum tolerated dose had been reached. No increase in the incidence of mononuclear cell leukemia in female rats was observed. This is in direct contrast to the results of the initial bioassay, although both studies contained a similar dosing group (600 mg/kg/day) at which the increased incidence was observed in the earlier bioassay. The 1997 repeat study results lend further weight to the conclusion of the initial study authors that the increase in incidence of MNCL was of equivocal biological significance.

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[6] NTP historical control data for F-344 rats show that MNCL occurs in 14 to 74 percent of control animals (Haseman, et al., 1998). The background incidence is highly variable and has more than doubled from about 1985 to about 2005 (Haseman,, et al., 1985; Thomas, et al., 2007). NTP decided to stop use of the F-344 strain, in part because of the high spontaneous incidence of MNCL in that strain (King-Herbert and Thayer, 2006; NTP BSC, 2007).

The incidences of transitional epithelial papilloma of the urinary bladder in female rats at 2 years were 1/50, 0/50, 0/50, and 2/50 in control, 300, 600, and 1200 mg/kg/day groups, respectively. The incidence of this lesion in the high-dose group, 2/50, is not statistically different from the incidence in the control group, 1/50; however, it is outside the reported range of 0-2% for untreated controls in NTP studies. The nonstatistically-significant increase in a benign neoplasia combined with the elevated (statistically-significant) hyperplasia of the urinary bladder epithelium was one of two elements forming the basis for NTP to conclude that BBP produced equivocal evidence of carcinogenic activity in female F-344 rats in this bioassay.

Unfortunately, NTP did not report whether urinary calculi were present in any of the female rats treated with BBP, especially those in the high-dose group developing bladder hyperplasia or tumors. Urinary bladder neoplasia in rodents and proliferative responses of the urinary bladder epithelium (like the hyperplasia reported in this study) have been observed in rats and mice following mechanical irritation by foreign bodies or calculi (Alison, et al., 1994). According to Alison, et al., "when administration of a chemical results in calculi and tumor formation, it is necessary to establish whether the (bladder) tumors are indeed induced by the chemical or occur as a secondary effect of the calculi. Low doses of compound that do not produce calculi do not produce tumors. This provides a simple example of a threshold effect for secondary carcinogenesis."

Pancreatic acinar cell adenomas were reported for the high-dose females (2/50 vs. 0/50 in controls). This incidence in high-dose female rats was not statistically different from the incidence in control animals and was within the range of NTP historical controls values. Nonetheless, because a pancreatic acinar cell tumorigenic effect occurred in male rats, NTP cited the finding of pancreatic acinar cell adenomas in female rats as the other reason (in addition to bladder effects described above) to conclude that there is equivocal evidence of carcinogenic activity of BBP in female rats in the assay. NTP's definition of "equivocal evidence of carcinogenic activity" is a study interpretation that shows a marginal increase of neoplasms that may be chemical related. Given that there was no real increase in neoplasms in female rats in this study, the designation of "equivocal evidence" appears to be questionable – "no evidence" is likely more appropriate.

In male rats, an increased incidence of pancreatic acinar cell adenoma (3/50, 2/49, 3/50 and 10/50 in control, 120, 240 and 500 mg/kg /day groups, respectively) and pancreatic acinar cell adenoma or carcinoma (combined) (3/50, 2/49, 3/50 and 11/50 in control, 120, 240 and 500 mg/kg /day groups, respectively) was observed in the high-dose group. These incidences were statistically significant. No difference in food consumption was reported for the high-dose male rats compared to control; however, the high-dose male rats in this study weighed less, on average, than control animals, suggesting that the caloric intake for the high-dose animals was greater (on a body weight basis) than for concurrent control animals. A possible dietary role in pancreatic carcinogenesis, as suggested in this study, was, in fact, demonstrated in the third NTP bioassay of BBP (NTP, 1997b). The findings of that study, a dietary restriction study, are described below in the next section.

It is known from published analyses of the NTP carcinogenicity database (Haseman, et al., 1985; Haseman and Rao, 1992) that for male F-344 rats a relationship can be shown between pancreatic acinar cell tumors and corn oil gavage treatment. The effect is not related to gavage

technique and appears to be sex-specific. The BBP cancer bioassay reported in 1997 (NTP, 1997a) was not a gavage study and did not involve the use of corn oil as a vehicle, but did provide an indication that dietary factors may play an important role in male rat pancreatic tumorigenesis, a role confirmed in the 1997 dietary restriction study with BBP (NTP, 1997b).

Moreover, a series of papers published beginning in 1997 identified a number of factors in addition to diet which influence the development of pancreatic lesions in the rat (Obourn, et al., 1997a; 1997b; Biegel, et al., 2001). These lesions included acinal cell hyperplasia and adenocarcinoma formation. The authors of those studies stated that any factor which can affect circulating steroid levels or cholecystokinin (CCK) levels, or cause overexpression of the CCK(A) receptor may increase the development of pancreatic acinar cell changes and lead to tumor formation in the rat. Accordingly, these factors, even if induced secondarily as a result of toxicity in another organ, can stimulate the development of pancreatic acinar cell pathology. In fact, Obourn (1997a) and Biegel, et al. (2001) concluded in a study of a peroxisome proliferating agent that a mild but sustained increase in CCK production secondary to liver changes (cholestasis) may be responsible for pancreatic acinar cell pathology, including tumors. These reports may have applicability to BBP and the findings of pancreatic acinar cell tumors in the NTP studies because of the ability of BBP to induce hepatic changes in the Fischer rat at dietary doses equivalent to the mid- and high-dose concentrations employed in the NTP studies, and the role of diet in the observation of pancreatic tumors (Monsanto, 1994).

The significance of secondary toxic effects and the role these may play in rat pancreatic pathogenesis should be part of the interpretation of the pancreatic acinal cell tumorigenic effects observed in male rats in the BBP studies. This is particularly the case since no other elevation in tumor incidence was observed in male rats in this study and since the pancreatic effect thought to be secondary to liver damage is considered to be species specific, *i.e.*, limited to the rat (Obourn, 1997a).

Finally, since focal abnormalities of acinar pancreatic cells (atypical acinar cell foci or nodules) are reported to occur spontaneously in rats at an incidence of zero to 75% in 24 month old rats (Woutersen, et al., 1991), the elevated incidence of focal hyperplasia of the pancreatic acinar cell in the high-dose males of the 1997 NTP bioassay should have been put into a broader perspective than provided by NTP. The authors concluded that there was "some evidence of carcinogenic activity" in male rats, based upon the increased incidences of pancreatic acinar cell adenoma and of acinar cell adenoma or carcinoma (combined). Given that the only neoplastic lesions or even preneoplastic lesions observed in male rats were pancreatic and occurred at very low incidences, and considering the mitigating factor of a dietary role in pancreatic tumor formation, NTP would have more properly categorized the results of this study as “equivocal evidence” of carcinogenic activity, *i.e.*, a study showing a marginal increase of neoplasms that may be chemical related.

C. NTP, 1997b: Evidence for role of diet in pancreatic tumors in male rats

The third NTP bioassay on BBP was conducted as part of an effort to compare the effect of *ad libitum* feeding versus dietary restriction on the outcome of chronic bioassays (NTP, 1997b). Male rats were dosed with 12,000 ppm BBP in their diet for 24 or 30 months; females

were dosed with 24,000 ppm for 24 or 32 months. Test groups included *ad libitum* fed control and treated rats, control and treated rats that received an amount of food so that mean body weight matched the mean body weight of the *ad libitum* dosed group (weight-matched), and control and treated rats that were maintained at about 85% of the body weight of the untreated controls in the *ad libitum* study (weight restricted).

An increase in the incidence of pancreatic acinar cell neoplasms was observed in BBP-treated *ad libitum* fed male rats compared to *ad libitum* fed and weight-matched controls. Interestingly, no increase was observed in restricted diet treated group after 2 years but acinar cell adenomas were observed in 3 animals at 30 months reinforcing the role of diet in the expression of this tumor following administration of BBP.

In female rats, a slight increase in urinary bladder neoplasms was observed, but only at a 32-month time point, in the restricted feed treated group. The increase was not statistically significant.

The incidences of MNCL in exposed males were statistically-significantly greater than those in the weight-matched controls but similar to the incidence in the controls fed *ad libitum* and within the historical control ranges for leukemia (all types) in untreated rats. The incidences of MNCL in exposed females were greater than those in the weight-matched controls but less than the incidence in the controls fed *ad libitum* and within the historical control ranges for leukemia (all types) in untreated rats. The incidences in weight-restricted male rats at 24 months were slightly higher than weight-restricted controls (54% v. 42%) and slightly lower (92% v. 94%) than weight-restricted controls at 30 months, but the differences were not statistically significant. The incidences in weight-restricted female rats at 24 months was slightly higher than weight-restricted controls (36% v. 32%) and significantly higher (78% v. 58%) than weight-restricted controls at 32 months. In evaluating the study results, NTP noted that all MNCL findings were within historical control incidence ranges for untreated rats, and NTP did not include MNCL in their pathology and statistical analyses of "significant or biologically noteworthy changes."

Mammary gland tumor incidences, both fibroadenoma and adenomas and carcinomas combined, were statistically-significantly reduced by BBP treatment compared to the *ad libitum* fed group and to the 24 and 30-month restricted feed control groups.

D. Short-term animal studies for carcinogenic response: Theiss, et al. (1977); Singletary, et al. (1997)

Theiss's group (Theiss, et al., 1977) showed that intraperitoneal injections to Strain A mice of BBP three times per week for 8 weeks (a standard tumor induction model) failed to produce a treatment-related increase in pulmonary tumors, the end point of the bioassay. Doses used in the study were high: 160, 400 and 800 mg/kg/injection, yielding a total dose of 3,840, 9,600 and 19,200 mg per animal, respectively. Twenty animals were used per dose level. Mice were held for 24 weeks following the final injection. All animals survived to study termination. No increase in pulmonary tumors occurred as a result of BBP treatment.

Singletary, et al. (1997) employed an initiation-promotion model for assessment of BBP tumorigenicity and DNA adduct formation. Mammary tumors were initiated with dimethylbenz[a]anthracene (DMBA) in groups of 27 female rats. DMBA was given by oral gavage at a dose of 31 mg/kg. BBP was administered orally via gavage at doses of 250 or 500 mg/kg. BBP was administered for 7 consecutive days prior to administration of DMBA. BBP inhibited total mammary tumor formation by 37% at each dose level. Adenocarcinoma formation was inhibited 60% and 70% at the low and high-dose of BBP, respectively.

Singletary, et al. (1997) also reports that BBP administration for 5 days via intraperitoneal injection (ip) at 100 and 500 mg/kg/day or oral gavage at 100 or 500 mg/kg/day reduced mammary DMBA-DNA adduct formation by 2% and 92% (ip) and 48% at 500 mg/kg/day by gavage.

Thus, these short-term studies provide no evidence that BBP is a carcinogen or co-carcinogen.

II. The Weight of the Evidence Strongly Indicates that BBP Is Not Genotoxic

BBP has been tested in a variety of *in vitro* and *in vivo* genetic toxicity assays for genetic toxicity endpoints and for the ability to induce morphologic transformation. The *in vitro* assays were conducted with bacterial, yeast and mammalian cell systems. *In vivo* assays were conducted in mice, rats and *Drosophila*. In most assays, BBP was negative; in the remainder results were equivocal. The great weight of the evidence indicates that BBP is not genotoxic.

The results of the BBP genotoxicity assays are summarized in Appendix B (Tables 1-4). More detailed discussion follows here.

A. In Vitro Assays

BBP was not mutagenic in the Ames *Salmonella* assay with and without activation (Litton Bionetics Inc., 1976; Rubin, et al., 1979; Kozumbo, et al., 1982; Zeiger, et al., 1985). It was also shown to be without genotoxic activity (mutation) in *E. coli* and was negative for DNA damage in *Bacillus* bacteria (Omari, 1976). When tested in eurokaryotic cells, BBP was negative for mutation in D4 yeast cells (Litton Bionetics Inc., 1976).

In the mouse lymphoma assay, BBP produced either negative (Litton Bionetics Inc., 1977; Hazleton Biotechnologies Company, 1986; Barber, et al., 2000), or equivocal responses (Myhr, et al., 1986; Myhr and Caspary, 1991). Testing in that assay system, the L5178Y Mouse Lymphoma cell line, was performed in multiple trials with and without exogenous metabolic activation and was conducted at BBP concentrations at or greater than the limit of BBP solubility in cell culture medium.

BBP did not produce *in vitro* transformation of Balb/c-3T3 cells (Litton Bionetics Inc., 1985; Barber, et al., 2000), nor did it produce transformation in Syrian hamster embryo cells (Le Boeuf, 1996). Primary cultures of Syrian hamster embryo cells retain significant innate

metabolic capability and transformation assays using these cells are typically performed without exogenous metabolic activation (Ashby, et al., 1985), as was the case with BBP testing.

In an assay for chromosomal aberrations and sister chromatid exchanges (SCE) in Chinese hamster ovary cells (Galloway, et al., 1987), there was slight evidence for a trend in an increase in SCE formation in one of two trials without activation, but no evidence for SCE formation in a trial with activation. There was no evidence for induction of chromosome aberration by BBP. The authors concluded that the study was negative for the induction of SCE and chromosome aberration.

B. In Vivo Assays

In vivo assays have also been negative or equivocal for evidence of genetic toxicity. A negative response was reported in assays for the induction of sex-linked recessive lethals in *Drosophila melanogaster* dosed by feed and also dosed by injection (Valencia, et al., 1985).

Results from mouse bone marrow tests examining induction of either sister chromatid exchanges (SCE) or chromosomal aberrations indicated weak responses (NTP, 1997a). A close look at the data shows that the results of these tests must be viewed with caution. Groups of 5 B6C3F1 male mice received a single intraperitoneal (ip) injection of 1250, 2500 or 5000 mg/kg BBP for evaluation of SCE in a single trial. The ip LD50 for mice (Swiss Webster) is 3160 mg/kg (Calley, et al., 1966). Information on test animal survival, weight gain or signs of systemic toxicity during the test period was not included in the NTP report of this study. However, SCE test data from the top dose (5000 mg/kg) were excluded by the investigators from analysis because of "a reduction in response," presumably due to excessive systemic toxicity. Two marrow cell harvest times were used, 23 and 42 hours post-dose. The number of cells scored per dose group was low: twenty-five marrow cells per animal (4 animals per group) were scored. There was no dose at either harvest time reported to have induced a statistically significant elevation in SCE formation. Despite, or perhaps because of, the extreme dose levels used (well into the systemically toxic range and in excess of the LD50) and the aggressive route of administration (intraperitoneal injection), there was no dose-response characteristic to the study data. The results were reported as positive for a trend in increased SCE formation without a significant increase in SCE levels in treated animals.

Similarly, mice treated with BBP as described above for the SCE study were evaluated for chromosomal aberration (NTP, 1997a). Ten male mice per group received an injection of 1,250, 2,500 or 5,000 mg/kg BBP and were evaluated at 17 or 36 hours post-dose for signs of chromosomal aberration in bone marrow cells. Fifty metaphase cells were examined for each animal; current guidelines for this type of study (OPPTS, 1998) require evaluation of 1000 cells per animal. No data were provided in the report concerning mortality, signs of systemic toxicity, etc., but a significant increase in chromosomal aberration was cited for the high dose group in each of the 17-hour harvests. There was no increase in aberrations in the 36-hour harvest. Although there was no dose-response relationship established for any of the three trials, a significant trend was reported for the 17-hour harvest trials. Without information on the condition of the high dose animals, and understanding that the dose employed in this group was nearly 60% above the LD50 for BBP by ip injection, interpretation of the results from the study is difficult and should be done with caution.

In contrast, negative results were reported by Ashby, et al. (1997) in a micronucleus assay in rats and by Bishop (1987) for a mouse (two species) dominant lethal mutation assay.

Thus, the weight of the evidence strongly indicates that BBP is not mutagenic or genotoxic. This evidence is summarized in Appendix B, Tables 1-4.

III. Other Animal Data Do Not Support a Concern that BBP is a Potential Carcinogen

Besides the data discussed above, OEHHA's compilation from its preliminary toxicology review cites data concerning estrogenic activity, plus two other mechanistic studies. These studies do not point to potential carcinogenicity for BBP. The weight of the evidence is that BBP is not estrogenic *in vivo*; further, *in vitro* estrogenicity studies such as those cited by OEHHA are not useful for evaluating the potential carcinogenicity of BBP. One of the mechanistic studies concerns activation of peroxisome proliferator-activated receptors (PPARs), a mechanism that is not relevant to human risk assessment. The other relies on an inaccurate interpretation of the BBP database.

A. The Weight of Evidence is That BBP is Not Estrogenic

OEHHA cites several *in vivo* studies on estrogenic activity and BBP. These papers and their relevance to human carcinogenicity assessment are the topic of an opinion written by Timothy Zacharewski, Ph.D., a leading expert on *in vitro* and *in vivo* models for estrogenicity and other endocrine modulating. Dr. Zacharewski's opinion is attached as Appendix C. It shows that the weight of the evidence is that BBP is not estrogenic.

Dr. Zacharewski discusses the need for a weight-of-evidence approach to assessing toxicity data and a critical assessment of data quality, all of which go beyond scoring positive and negative results of a group of studies. While properly qualified *in vitro* assays can be useful in identifying chemicals that interact with the estrogen receptor, they do not replicate *in vivo* conditions and have a poor record of predicting *in vivo* responses. *In vitro* studies are limited by the absence of pharmacokinetic and pharmacodynamic processes that occur in the intact organism. "Therefore, in order to assess potential impacts on the endocrine system, the chemical must be tested in an intact *in vivo* model" (Zacharewski, Appendix C, p. 3).

In several *in vitro* assays of estrogenicity, including proliferation of MCF-7 breast cancer cells, BBP has given very weak positive responses – more than a million times weaker than 17β-estradiol, the predominant female sex steroid. Dr. Zacharewski discusses factors that limit the usefulness or reliability of these studies for evaluation of BBP estrogenicity. Further, and most importantly, "BBP is not estrogenic *in vivo* based on the uterotropic assay, the gold standard for assessing the estrogenicity of a chemical" even at dose levels far above those of human exposures (Zacharewski, Appendix C, p. 4).

B. In Vitro Studies of Potential Estrogenicity Are Not Useful for Evaluating the Potential Carcinogenicity of BBP

Dr. Zacharewski's opinion also discusses the value of *in vitro* assays for evaluating the potential carcinogenicity of BBP. In addition to the inherent limitations on the value of *in vitro* assay for evaluating estrogenicity summarized above, his points include the following:

- In whole organisms, BBP is readily metabolized to monoester metabolites. These metabolites are negative for estrogenic activity in the E-Screen and yeast-based assays;
- BBP concentrations in the studies cited by OEHHA were extremely high – well beyond the aqueous solubility of BBP – confounding data interpretation;
- The E-Screen assay (MCL-7 cell proliferation) is prone to false positives;
- In a collagen assay, unlike 17β-estradiol, BBP did not cause formation of duct-like or solid mass structures;
- BBP significantly reduced *in vivo* formation of mammary DNA adducts and mammary adenocarcinomas induced by DMBA; and
- There is no reported evidence of BBP causing mammary carcinogenesis in high dose multigenerational reproductive and developmental toxicity studies.

Dr. Zacharewski concludes that "the weight of evidence indicates that BBP is not carcinogenic via an estrogenic mode of action" (Zacharewski, Appendix C, p. 6).

C. Other Mechanistic Data Do Not Support a Concern of Potential Carcinogenicity of BBP

OEHHA cites two publications it identifies as "other mechanistic considerations" – Hurst and Waxman (2003) and Agas, et al. (2007). The studies do not provide substantial support for a concern of potential carcinogenicity of BBP in humans.

Hurst and Waxman (2003) characterize activation of rodent and human peroxisome proliferator-activated receptors PPARα and PPARγ. These receptors are associated with a variety of cellular activities and phthalate monoesters are ligands for these receptors. Activation of PPAR receptors in rodents by some phthalate esters (but not BBP) is responsible for the development of liver cancer in those species. Based on rodent models, Hurst and Waxman argue that activation of PPAR receptors in humans, particularly PPARγ, may lead to adverse consequences including cancer. Other, data, however, do not support this theory.

A 2004 article by Bility, et al. investigated rodent and human PPARα, $PPAR_{\beta}$ and PPARγ activation by phthalate monoesters including monobutyl phthalate and monobenzyl phthalate. Bility, et al. showed that, among the common phthalate monoesters, those derived from BBP are the least or next-to-least potent activators of PPAR. More importantly, Bility, et al. showed major species differences in receptor activation between mouse, rat and human PPAR. Human PPAR receptors were sensitive to activating ligands including BBP metabolites compared to rodent PPAR. This lack of receptor sensitivity is mirrored in significant differences in the response of humans and rodents to phthalates and other peroxisome proliferators – humans and other primates are more refractory to phthalates than rodents (*see, e.g.*, Klaunig, et al., 2003). Bility, et al. also point out that in animal models activation of PPARγ can be both a potentiator of

carcinogenic effects and an inhibitor of carcinogenic effects. The findings reported by Bility, et al. suggest that any PPAR-based mechanistic consideration of BBP as a carcinogen, especially a human carcinogen, is premature.

Agas, et al. (2007) report on *in vitro* studies showing actin redistribution in a rat osteoblast cell line exposed to BBP. Standard techniques to assess gene activation and protein synthesis showed a decrease in actin synthesis in the presence of BBP and, following removal of BBP from the culture, an increase in actin synthesis. The authors interpret this as triggering an overall potentiation of cell growth. Intracellular localization of actin also was demonstrated. The authors reported that BBP increased osteoblast "viability" and culture growth (cell number) but relied on a single indirect technique – an increase in a metabolic breakdown product (formazan) – to assess both parameters. They also provided a qualitative indication that cyclin D3 is increased *in vitro* by BBP. Without presenting any additional empirical data the authors speculated that BBP could: affect translocation of fibroblast growth factor 2 (FGF) into the nucleus; alter DNA synthesis, cell proliferation and cell cycle progression since "some FGF's" are reported to regulate DNA synthesis; disrupt regulation of the D-cyclins since "D-type cyclins probably serve as integrators of growth factor-induced signals;" contribute to oncogenesis because of aberrant protein expression; and effect all of this through stimulation of cyclin D3 because "cyclins are molecules implicated in various cancers."

The authors support these hypotheticals by erroneously citing Zacharewski (1998) to claim that BBP mimics 17β-estradiol and then intimating that BBP is the physiologic equivalent of 17β-estradiol, a carcinogen. From this, Agas, et al. appear to apply findings on 17β-estradiol to BBP. As pointed out by Dr. Zacharewski in his opinion (Appendix C), summarized above, BBP exhibits only very weak *in vitro* estrogenicity and does not induce significant activity *in vivo*. Thus, the foundation for the supposition of Agas, et al. is undercut, and this paper does not provide a reliable basis for hypothesizing potential carcinogenicity of BBP.

IV. IARC and Other Authoritative Reviews of BBP Carcinogenicity Data Have Concluded There Is Low Concern for Human Carcinogenicity

The two-year cancer bioassays on BBP were conducted by the National Toxicology Program. NTP is responsible for publishing the Report on Carcinogens (ROC), listing chemicals NTP determines to be known or reasonably anticipated to cause cancer in humans. NTP often selects chemicals for evaluation for ROC listing from chemicals it has tested. NTP has never formally considered BBP for ROC listing,[7] indicating that it does not find the bioassay findings to raise significant concern about the potential carcinogenicity of BBP.

The BBP data have been reviewed by several other authoritative agencies. In each case, the conclusion indicates low concern for human carcinogenicity from BBP exposure. In

[7] Appendix C of the ROC (NTP, 2011) is "Substances Reviewed but Not Recommended for Listing in the Report on Carcinogens." BBP is not listed there, indicating that NTP has never considered BBP for listing.

accordance with the findings of these other careful agency reviews, BBP should be given a low priority for development of hazard identification materials.

The International Agency for Research on Cancer (IARC) classified BBP as Group 3 (not classifiable as to human carcinogenicity) in 1982 (IARC, 1982). In 1999, IARC reevaluated BBP (including consideration of the three NTP assays) and reconfirmed the Group 3 classification, finding the evidence in experimental animals to be limited (IARC, 1999).

OEHHA previously evaluated the carcinogenicity data for BBP (including the first two NTP bioassays) and determined there was a low level of carcinogenicity concern for BBP. OEHHA therefore gave BBP a low priority for further evaluation for listing under California Proposition 65 (OEHHA, 1997). The primary difference in the BBP database since that time is the addition of the third NTP bioassay, which indicates a role for diet in the pancreatic acinar cell tumors observed in male rats.

In 1998, Health Canada and Environment Canada produced an assessment of BBP toxicology, which then became the basis for an International Programme on Chemical Safety (IPCS)[8] Concise International Chemical Assessment Document (CICAD) (IPCS, 1999). These parties concluded:

> Therefore, BBP has induced an increase in pancreatic tumours primarily in one sex of one species, the full expression of which was prevented in a dietary restriction protocol, and a marginal increase in bladder tumours in the other sex, which was delayed upon dietary restriction. The weight of evidence of genotoxicity is negative, and, although weak clastogenic potential cannot be ruled out, available data are consistent with the compound not interacting directly with DNA. On this basis, BBP can be considered, at most, possibly carcinogenic to humans, likely inducing tumours through a non-genotoxic (although unknown) mechanism. (IPCS, 1999, Exec. Summ.)

Most recently, the European Commission of the European Union issued its European Union Risk Assessment Report for BBP (ECB, 2007). In that document the European Commission concluded that "BBP may be a borderline case between no classification (not a carcinogen) and Carcinogen Category 3 (available information is not adequate for making a satisfactory assessment). However, due to the lack of genotoxic effects no classification is proposed."

Thus, several separate assessments by agencies with expertise in carcinogenicity risk assessment have found BBP to pose low concern for carcinogenic risk to humans. Accordingly, BBP should be given a low priority for development of hazard identification materials.

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[8] IPCS is a joint venture of the United Nations Environment Programme, the International Labour Organisation, and the World Health Organization

V. Biomonitoring Data for BBP Show that Human Exposures Are Extremely Low

Substantial urinary metabolite data for BBP are available in the National Health and Nutrition Examination Survey (NHANES) database of the Centers of Disease Control and Prevention (CDC). Using the method of David (2000) and Kohn, et al. (2000), this data can be converted to provide an estimate of the exposure to BBP, using the following equation.

$$DI = [UC \times CE / (FUE \times 1000)] \times [MWd/MWm]$$

DI == daily intake of BBP (μg/kg/day)
UC = creatinine corrected urinary metabolite concentration (μg/kg) (from CDC, 2011)
CE = creatinine excretion rate (mg/kg/day) (for adults, 20 mg/kg/day from David, 2000 and Kohn, et al., 1999; 25 mg/kg/day for children ages 6-11, extrapolated from Wittassek, et al., 2011)
FUE = fractional urinary excretion rate of the metabolite (unitless) (0.73 from Anderson, et al., 2001)
MWd = molecular weight of BBP (312)
MWm = molecular weight of the monobenzyl phthalate (MBzP) (256)[9]

Using the most recent NHANES data reported by CDC (CDC, 2011, 2007-2008 samples), the geometric mean exposure of the total population is 0.3 ug/kg/day and the 95th percentile exposure is 2 ug/kg/day. Geometric mean and 95th percentile exposures for children aged 6-11 are 1.1 and 6.8 ug/kg/day, respectively. Of note, exposure levels have been trending downward.

In the 1997(a) NTP study the lowest BBP dose administered *ad libitum* that produced evidence of tumor formation (albeit equivocal evidence) was 12,000 ppm and 24,000 ppm for male and female rats, respectively. This equates to approximately 500 mg/kg/day in males and 1,200 mg/kg/day in females. As discussed in section I.B., above, treatment at these levels produced equivocal evidence of pancreatic acinar cell adenomas and carcinomas in males but did not produce statistically-significant tumors in females. These BBP dose levels in rats are 250,000 to 4,000,000-fold above the CDC NHANES levels for adult human exposure and 73,000 to 1,100,000-fold above the CDC NHANES levels for children's exposure.

Thus, the biomonitoring data for BBP indicate that there is little concern of health effects from human exposures to BBP. Again, this indicates that BBP should be a low priority for preparation of hazard identification materials.[10]

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[9] Note: It is inappropriate to use monobutyl ester concentrations to estimate BBP exposure, as they may be sourced in dibutyl phthalate exposures.

[10] We note that in 1997, OEHHA gave a low priority ranking to BBP even though its potential for human exposure was rated "high" (OEHHA, 1997). The subsequent biomonitoring data confirm that a low priority ranking for BBP is appropriate.

CONCLUSION

The foregoing demonstrates that the concern for BBP induced carcinogenicity is very low. Taken as a whole, the bioassay data for BBP do not reveal any strong tumor responses, as shown by the lack of response in mice; inability to reproduce the leukemia response in female rats; absence of rare, or early onset tumors; and the low incidences and influence of the diet or length of study on the pancreatic tumors in male rats or the urinary bladder tumors in female rats. The genotoxicity information on BBP is overwhelmingly negative. The weight of evidence is that BBP is not estrogenic, and that *in vitro* estrogenicity studies are not useful for evaluating the potential carcinogenicity of BBP. Other mechanistic data do not provide a basis for concern for BBP carcinogenicity, because they involve a mechanism not relevant to humans, or are grounded in an erroneous interpretation of the data.

The NTP, which conducted the cancer bioassays on BBP, has not considered BBP for listing in its Report on Carcinogens. Prior evaluations of the BBP database by other expert bodies have concluded that the potential for BBP to cause carcinogenicity in humans is, at most, marginal. Further, biomonitoring data demonstrate that human exposures to BBP are extremely low.

For all these reasons, BBP should be given a low priority for development of hazard identification materials under Proposition 65.

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APPENDIX A

Summary Comments on OEHHA Preliminary Toxicological Evaluation Compilation

OEHHA PRELIMINARY TOXICOLOGICAL REVIEW	EVIDENCE FOR POTENTIAL CARCINOGENICITY
Long-term feeding studies of 103-week studies in male and female B6C3F1 mice: NTP (1982) - No treatment-related tumor findings in males or females	No evidence for carcinogenicity
103-week studies in male and female *ad libitum* fed F344/N rats: NTP (1982) - Increase in mononuclear cell leukemia (by pairwise comparison and trend) in females	MNCL finding not reproduced in 1997(a) NTP bioassay. NTP authors (and others) state that MNCL is common in female rats, has a high spontaneous incidence rate, and is not a relevant lesion in humans.
103-week studies in male and female F344/N rats: NTP (1982) - No treatment-related tumor findings in males, but the study was judged inadequate due to high mortality in treated animals.	No evidence for carcinogenicity (early termination of males)
105-week studies in male and female F344/N rats: NTP (1997a) - Increase in pancreatic acinar cell adenoma, and adenoma and carcinoma (combined) (by pairwise comparison and trend) in males	A dietary role for pancreatic carcinogenesis was demonstrated in a third NTP study of BBP (NTP, 1997b).
105-week studies in male and female F344/N rats: NTP (1997a) - urinary bladder transitional epithelial papilloma (significant compared to historical controls) in females	Bladder tumors were not statistically-significantly elevated above controls in this study
24- or 30- or 32-month studies in male and female F344/N rats on restricted or *ad libitum* diets: NTP (1997b) - Increase in urinary bladder carcinoma, and carcinoma and papilloma (combined) in females on restricted diet	Slight increase in urinary bladder neoplasm but only at 32 months on restricted diet and none were statistically-significantly elevated above controls at $P<0.05$
24- or 30- or 32-month studies in male and female F344/N rats on restricted or *ad libitum* diets: NTP (1997b) - Increase in pancreatic adenoma in males fed *ad libitum* (by pairwise comparison, for either *ad libitum* or weight-matched controls	No increase in pancreatic tumors in weight-restricted rats at 2 years. This reinforces the role of diet in pancreatic tumorigenesis.

OEHHA PRELIMINARY TOXICOLOGICAL REVIEW	EVIDENCE FOR POTENTIAL CARCINOGENICITY
24- or 30- or 32-month studies in male and female F344/N rats on restricted or *ad libitum* diets: NTP (1997b) - Increase in mononuclear cell leukemia in males and females fed *ad libitum* (pairwise comparison with weight-matched controls)	The incidence of MNCL in BBP-treated animals was within the range of historical controls for this strain and sex rat and not elevated above *ad libitum*-fed controls. NTP authors (and others) state that MNCL is common in female rats, has a high spontaneous incidence rate, and is not a relevant lesion in humans and did not consider MNCL in this study as biologically noteworthy.
24- or 30- or 32-month studies in male and female F344/N rats on restricted or *ad libitum* diets: NTP (1997b) - No treatment-related tumor findings in males on restricted diet	No evidence for carcinogenicity
Short-term intraperitoneal injection study in mice 24-week study in Strain A mice (injected 3 times/week for 8 weeks): Theiss, et al. (1977), as reviewed by NTP (1982) - No treatment-related increase in pulmonary tumors	No evidence for carcinogenicity
Short-term co-carcinogenicity 16-week study in female Sprague-Dawley rats (gavaged 7 times/week for one week, followed by a single dose of dimethylbenz[a]anthracene): Singletary, et al. (1997), as described in IARC (1999, p. 118) - No co-carcinogenic effects observed	BBP reduced the incidence of DMBA-induced breast tumors
Genotoxicity Review: NTP (1997a, pp. 7-8, 50); IARC (1999, pp. 123-124) - Salmonella reverse mutation assays (negative) - Drosophila melanogaster sex-linked recessive lethal mutation assays (negative) - Mouse lymphoma cell mutation assay (negative) - Sister chromatid exchange (SCE) in Chinese hamster ovary (CHO) cells (negative) chromosomal aberrations in CHO cells (negative) - SCE and chromosomal aberrations in mouse bone marrow cells *in vivo* (positive)	*See* Appendix B, Tables 1-4 for comprehensive summary of genetic toxicity studies completed on BBP. The results are overwhelmingly negative for genetic toxicity.
Estrogenic activity	*See* Opinion of Dr. Timothy Zacharewski, Appendix C, concluding that "the weight of evidence indicates that BBP is not carcinogenic via an estrogenic mode of action."
Mechanistic considerations; Hurst and Waxman (2003) - Showed weak peroxisome proliferation inducing activity	There are significant species differences in activation of PPARα and PPARγ by phthalate monoesters. Literature supports activation of PPARγ as being both carcinogenic and anti-carcinogenic. (Bility, et al., 2004)

OEHHA PRELIMINARY TOXICOLOGICAL REVIEW	EVIDENCE FOR POTENTIAL CARCINOGENICITY
Mechanistic considerations; Agas, et al. (2007) - Influenced actin distribution and cell proliferation in rat osteoblasts and increased levels of Cyclin D3 at G1 to S transition.	Authors offer support for BBP role in *in vitro* actin utilization and offer evidence for BBP *in vitro* induction of cyclin D but do not provide data to support a cancer mode-of-action for any tumor type.
IARC Reviews	IARC, Health Canada, OEHHA (1997) and the European Union have reviewed the carcinogenicity database for BBP and concluded that there should be no or low priority assigned

APPENDIX B

Genetic Toxicity Tables

Genetic Toxicity Table 1 *In Vitro* Gene Mutation Tests in Prokaryotic cells		
Study Design	Results	Reference
Salmonella typhimurium Strains TA98, TA100, TA1535, TA1537 and TA1538 With and without S-9 exogenous metabolic activation	Negative for mutation in all strains tested	Litton Bionetics, 1976
Salmonella typhimurium Strains TA98, TA100, TA1535, TA1537 and TA1538 With and without S-9 exogenous metabolic activation	Negative for mutation in all strains tested	Monsanto, 1976; Rubin, 1979; Kozumbo, 1982; Zeiger, 1985
Salmonella typhimurium Strains TA 98, TA 100, TA 1535, TA 1537 Four trials; with and without S-9 rat or hamster liver cell exogenous metabolic activation	Negative for mutation in all strains, all trials tested except one trial TA100 gave equivocal results without activation	NTP, 1997
Escherichia coli	Negative for gene mutation	Kurata, 1975 as cited in Omori, 1976
Bacillus subtilis	Negative for DNA repair	Kurata, 1975 as cited in Omori, 1976
Saccharomyces cerevisiae, Strain D4 With and without S-9 exogenous metabolic activation	Negative for mutation	Litton Bionetics, 1976

Genetic Toxicity Table 2 *In Vitro* Gene Mutation Tests in Mammalian Cells		
Study Design	Results	Reference
L5178Y Mouse lymphoma cells With and without mouse liver S-9 exogenous metabolic activation	Negative for mutation in all concentrations tested up to the limit of BBP solubility in cell culture medium	Litton Bionetics, 1977; Hazleton, 1986
L5178Y Mouse lymphoma cells With and without mouse liver S-9 exogenous metabolic activation	Equivocal for point mutation	Myhr, 1986; Myhr and Caspary, 1991
L5178Y Mouse lymphoma cells With (four trials) and without (two trials) rat liver S-9 exogenous metabolic activation	Negative for mutation in all concentrations tested up to the limit of BBP solubility in cell culture medium	NTP, 1997a

Genetic Toxicity Table 3 *In Vitro* Chromosome Damage and Morphologic Cell Transformation Tests in Mammalian Cells		
Study Design	Results	Reference
Chinese hamster ovary cell One trial with and two trials without rat liver S-9 exogenous metabolic activation for evaluation of sister chromatid exchange (SCE) figures	Negative -- Positive trend for SCE in first trial without activation but no significant increase in SCE at any test concentration; no increase in SCE at any test concentration or positive trend in second trial without activation or trial with activation. Author concluded study was negative for the induction of SCE.	Galloway, 1987
Chinese hamster ovary cell With and without rat liver S-9 exogenous metabolic activation	Negative -- No induction of chromosome aberration was detected with or without activation	Galloway, 1987
Syrian hamster embryo cells	Equivocal -- Morphologic cell transformation did not occur at 24 hours in any concentration tested within the range of BBP solubility in the cell culture medium; a positive response was observed following 7 days in culture.	Le Boeuf, 1996
BALB/3T3 cells	Negative -- Morphologic cell transformation did not occur at any concentration tested within the range of BBP solubility in the cell culture medium	Monsanto, 1985

Table 4 *In Vivo* Genetic Toxicity Tests		
Study Design	Results	Reference
Drosophila melanogaster dosed in feed at 10,000 or 50,000 ppm BBP or by injection at 500 ppm	Negative for sex-linked recessive lethal mutation	Valencia, 1985
Mouse, CD-1 and $B_6C_3F_1$ 400-4560 mg/kg/day subcutaneously for three days	Negative for dominant lethal mutation	Bishop, 1987
Male $B_6C_3F_1$ mice intraperitoneal injection of 1250, 2500 or 5000 mg/kg	Positive trend for SCE when top dose level was excluded from analysis of 23 hour cell harvest and for SCE for 48 hour cell harvest	NTP, 1997a
Male $B_6C_3F_1$ mice intraperitoneal injection of 1250, 2500 or 5000 mg/kg	Equivocal -- Positive trend for chromosomal aberration at 17 hour cell harvest; No positive trend (or significant elevation) in chromosome aberration at 36 hour harvest	NTP, 1997a
Female Alpk:AP_fSD (AP) rats receiving BBP in their drinking water at approx. 182.6 mg/kg/day during gestation and lactation (approx. 45 days)	Negative -- No induction of micronuclear bodies in bone marrow smears; Negative micronucleus test.	Ashby, 1997

APPENDIX C

Opinion of Timothy R. Zacharewski, Ph.D.

Expert Report on the Estrogenic Activities of Butylbenzyl Phthalate (BBP)

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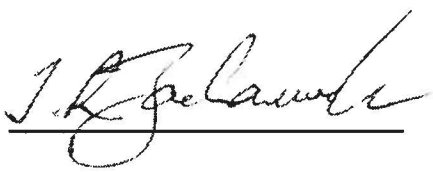

September 19, 2011

Introduction

I have been asked by the Ferro Corporation to comment on the "Other relevant data - Estrogenicity activity" studies in the Office of Environmental Health Hazard Assessment (OEHHA)'s Butyl Benzyl Phthalate (BBP) background document that the Carcinogen Identification Committee (CIC) will consider at its Oct. 12-13, 2011 meeting. I am a Distinguished College of Natural Sciences Professor of Biochemistry & Molecular Biology and member of the Center for Integrative Toxicology at Michigan State University. I obtained my PhD in toxicology at Texas A&M University in the area of *in vitro* toxicology and obtained additional training in molecular biology and nuclear receptors in the laboratory of Professor Pierre Chambon (LGME-CNRS, Strasbourg, France) as a Medical Research Council of Canada Post Doctoral Fellow. I have 25+ years of research experience investigating the mechanisms of toxicity of environmental contaminants and industrial chemicals including the examination of estrogenic endocrine disruptors using *in vitro* and *in vivo* models. To date, my laboratory has published more than 100 peer-reviewed primary papers. I have also participated in numerous invited national and international workshops on *in vitro* screening assays and served on advisory committees reviewing the health risks of endocrine disruptors. This includes serving on the U.S. Environmental Protection Agency's (EPA) Endocrine Disruptor Screening Program (EDSP) review committee and on the National Toxicology Program (NTP) Center for the Evaluation of Risks to Human Reproduction committee that reviewed 6 high production volume phthalate esters including butylbenzyl phthalate (BBP). I am currently on leave from Michigan State University and placed on special assignment at EPA's National Center for Environmental Assessment (NCEA) investigating the use of high throughput *in vitro* assay and omics data in risk assessment as an Oak Ridge Institute for Science and Education (ORISE) Faculty Fellow. The following comments represent my own opinions and do not necessarily reflect the views or policies of the US EPA. For the purposes of this report, I am acting independently of Michigan State University and EPA.

Summary

This report outlines my opinions on the value of *in vitro* assays in assessing the estrogenic activities of chemicals. I describe their strengths and weaknesses as well as discuss specific technical issues that must be considered when critically assessing data from *in vitro* assays. Most importantly, the predictability of *in vivo* responses and the use of *in vitro* data in risk assessment are described. These principles are applied when evaluating the *in vitro* estrogenicity of BBP and its potential human carcinogenicity.

Although *in vitro* assays can be useful to identify chemicals that interact with the estrogen receptor and to elucidate mechanisms of action, they do not replicate *in vivo* conditions. Overall, *in vitro* assays have a poor record of predicting *in vivo* responses, especially for complex diseases such as breast cancer. Consequently, it is my opinion that *in vitro* assays are not useful for evaluating the potential carcinogenicity of BBP.

Endocrine Disruption Screening

Endocrine hormones, including steroids, regulate diverse physiological processes such as reproductive tract development, fertility, energy balance and behavior. Imbalances in hormones can also contribute to complex diseases such as cancer and diabetes as well as compromise reproductive fitness and development (e.g., reduced sperm counts, cyrptorchidism). In response to public concern regarding the possible disruption of the endocrine system following exposure to drugs, chemicals, natural products and environmental contaminants, the U.S. Environmental Protection Agency (EPA) established the Endocrine Disruptor Screening Program (EDSP) in compliance with the Food Quality Protection Act and the Safe Drinking Water Act Amendments of 1996. This requires EPA to test all food contact chemicals

and any chemical found in drinking water for effects similar to those elicited by female hormones (estrogens) and gives EPA the authority to screen for other endocrine effects (e.g., androgen, thyroid) in humans and wildlife (www.epa.gov/endo).

EDSP uses a two-tiered approach to determine the potential for chemicals to cause endocrine disruption in humans and wildlife. The Tier 1 Screening battery, adopted by EPA based on recommendations from the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), consists of a battery of complementary *in vitro* and short-term *in vivo* assays designed to identify chemicals affecting the estrogen, androgen and/or thyroid hormone systems through any of several recognized modes of action. This includes using assays that measure *in vitro* receptor binding and/or transactivation (estrogen and androgen receptor), inhibition of aromatase activity (estrogen biosynthesis), frog metamorphosis (thyroid activity), effects on fish (estrogen and androgen effects), male development (Hersberger assay, androgen and anti-androgen effects), pubertal female development (estrogen and thyroid activity), pubertal male development (androgen/anti-androgen and thyroid), uterine response (estrogen effects) and 15-day adult intact male responses (anti-androgen and thyroid activity). Data from these assays is then used in a weight of evidence (WoE) approach to determine which chemicals in Tier 1 Screening warrant further examination in Tier 2 Testing to generate the data needed to support a risk assessment (www.epa.gov/endo).

Tier 2 Testing *in vivo* assays assess whole organism effects and provide apical, as well as mechanistic, information from one or multiple endpoints within the assay. Tier 2 Testing consists of *in vivo* tests in males and females with an intact hypothalamic-pituitary-gonadal axis, using multiple pathways of exposure, exposures at different life-stages and exposure to various taxa to further identify and characterize chemical induced interactions with the endocrine system that can be used in a risk assessment. Note that Tier 2 tests are designed to quantify dose-response relationships in the larger context of toxicity and potential adversity that may involve interactions with other biological systems (e.g., neurological, immunological, hepatic, renal, and cardiovascular). Therefore, regulatory action is based on Tier 2 Testing (www.epa.gov/endo).

For the EDSP, weight-of-evidence evaluation (WoE) is a prescribed process where potentially relevant studies are critically assessed for data quality (www.epa.gov/endo). More specifically, potential compound effects, mode of action (MOA), and assay performance are evaluated. This goes beyond assessing positive and negative results within and between studies. Critical scientific assessment of the entire body of available data is considered to account for consistency, coherence, and biological plausibility. EPA continues to refine its WoE approach for use in risk assessments of chemicals suspected of causing toxicity, and especially cancer. Most important is the use of expert judgment formed through the scientific process, a current understanding of toxicity mechanisms, and knowledge of complementary fields (*e.g.*, developmental, reproductive, neurological and immunological toxicology, pharmacokinetics/ pharmacodynamics) (Borgert *et al.*, 2011a; Borgert *et al.*, 2011b). The concept of using complementary *in vitro* and *in vivo* assays to inform risk assessment and regulatory decision making also appears to gaining acceptance among stakeholders (Hartung and Daston, 2009). Consequently, these same principles should be applied when evaluating the potential carcinogenicity of BBP in humans.

Limitations of *In Vitro* Assays

The endocrine system involves the integration of signals across multiple nodes (i.e., organs, tissues) throughout an organism (e.g., hypothalamic-pituitary-gonadal axis), which can be profoundly affected by the absorption, distribution, metabolism and excretion (ADME) of an endocrine disruptor. Interactions between different cell types and organs at different developmental stages can also affect a chemical's behavior. Therefore, in order to assess potential impacts on the endocrine system, the chemical must be tested in an intact *in vivo* model (Ankley *et al.*, 1998; Gray *et al.*, 1997; Spielmann *et al.*, 1998; Zacharewski, 1998; Zacharewski, 1997). Nevertheless, data from *in vitro* assays, when appropriately qualified, can also be informative regarding potential sites of action and mechanism of

action. Ideally, a comprehensive and complementary battery of assays should be used to avoid false positives and negatives, since estrogens and estrogenic endocrine disruptors can elicit species-, tissue-, cell-, and response-specific effects. Moreover, despite the conservation of function and modes of action of endocrine systems between species, there are significant differences that can dramatically alter the activity of a chemical. Therefore, *in vitro* assays used to assess endocrine disrupting activity should be human-based, and reflect human ADME characteristics, when possible. For example, rodent models and *in vitro* assays lack sex hormone binding globulins (SHBG) that are present in human serum (Hammond, 2011; Hammond and Bocchinfuso, 1996). SHBG is an estrogen inducible protein present in serum that binds estrogens and estrogenic chemicals to regulate their bioavailability and metabolic clearance (Ankley, et al., 1998; Gray, et al., 1997; Spielmann, et al., 1998; Zacharewski, 1998; Zacharewski, 1997).

In vitro assays also lack metabolic capabilities to bioactivate a proestrogenic chemical to its estrogenic metabolite or neutralize it through metabolism and eventual excretion. It is extraordinarily difficult to replicate the pharmacokinetic (e.g., metabolism) and pharmacodynamic (e.g. SHBG) interactions that are important for proper endocrine function, especially during development (Ankley, et al., 1998; Gray, et al., 1997; Spielmann, et al., 1998; Zacharewski, 1998; Zacharewski, 1997). Differences between species further confound data interpretation. For instance, humans preferentially metabolize BBP to mBzP (73% on a molar basis) over mBP (6% on a molar basis) (Anderson *et al.*, 2001). In contrast, rat metabolism of BBP yields 16% mBzP and 44% mBP (Anderson, et al., 2001). Consequently, due to their poor record of predicting *in vivo* activity, *in vitro* assay results for endocrine disrupting activities can only be used in a WoE approach to rank and prioritize chemicals that warrant additional *in vivo* Tier 2 Testing. It should also be noted that *in vitro* assay results are not used by the EPA for risk assessment. EPA also does not use data from other *in vitro* based screening programs such as ToxCast (Dix *et al.*, 2007) and Tox21 (Shukla *et al.*, 2010), for the purposes of risk assessment.

Assessment of *In Vitro* Studies Examining the Estrogenicity of BBP

The Office of Environmental Health Hazard Assessment (OEHHA) cites several papers under "Other relevant data – Estrogenic activity" that suggest BBP exhibits estrogenic activity based on *in vitro* data. For example, several papers are cited that report BBP binds to the estrogen receptor and induces ER-mediated effects such as the proliferation of human MCF-7 breast cancer cells. BBP is reported to be weakly estrogenic (>10^6 times weaker than 17β-estradiol, the predominant female sex steroid) in several *in vitro* assays (e.g., competitive estrogen receptor (ER) binding assays, ER-mediated gene expression in mammalian cells, ER-mediated reporter gene assays in mammalian cells, ER-mediated activity in yeast cells). However, BBP is not estrogenic *in vivo* based on the uterotropic assay, the gold standard for assessing the estrogenicity of a chemical (Brady *et al.*, 2000; Ryu and Kim, 2006; Zacharewski, 1997; Zacharewski *et al.*, 1998). These studies included doses that far exceed human exposure levels (e.g., daily doses ranging from 20 mg/kg to 2000 mg/kg per day for three consecutive days). In addition, estimated average drinking water exposures of 182.6 ug/kg/d to pregnant dams during gestation (gestational days 1-20) and from postnatal day 15 onward, had no effect on pup uterine weights (Ashby *et al.*, 1997b). Other sensitive *in vivo* markers of estrogen exposure including vaginal epithelial cell cornification (Zacharewski, et al., 1998), induction of uterine vascular permeability (Milligan *et al.*, 1998), and the differential expression of estrogen responsive uterine genes (Hong *et al.*, 2005), were also not affected by BBP.

Several factors must be considered when evaluating these *in vitro* studies. The first is that BBP is readily metabolized by non-specific esterases to monobutyl (mBP) and monobenzyl (mBzP) phthalate metabolites (Kayano *et al.*, 1997; Mentlein and Butte, 1989; Mentlein *et al.*, 1980). Consequently, mBP and mBzP should be the test chemicals used for *in vitro* assays. Interestingly, several of the cited reports indicate that mBP and mBzP are negative for estrogenic activity in the E-Screen and yeast-based assays (Harris *et al.*, 1997; Hashimoto *et al.*, 2003; Okubo *et al.*, 2003; Picard *et al.*, 2001). The concentrations used in the studies cited by OEHHA are also extremely high. Results obtained with doses exceeding 10

uM should be viewed with skepticism due to the insolubility of BBP in aqueous solutions (Moore, 2000). Precipitates would result in cells experiencing much higher concentrations than those nominally applied, which would confound data interpretation, and possibility cause cell toxicity. The Fernandez and Russo (Fernandez and Russo, 2010), Kang and Lee, (Kang and Lee, 2005), Kim et al., (Kim *et al.*, 2004), and Hashimoto et al., (Hashimoto, et al., 2003) studies all used BBP concentrations in excess of 10 uM, and even concentrations as high as 1 mM.

Of greatest concern are results derived from the E-Screen assay (Jones *et al.*, 1998; Welshons *et al.*, 1992; Zacharewski, 1997). Numerous studies have demonstrated that MCF-7 cell proliferation is highly variably and elicits a modest response (Picard, et al., 2001). This assay is also prone to false positives as a wide variety of chemicals and other treatments induce proliferation (Jones, et al., 1998; Welshons, et al., 1992). Assay reproducibility is problematic since several factors such as clone selection, culture conditions, serum lots and cell density influence proliferation (Jones, et al., 1998; Welshons, et al., 1992). As a result of these concerns, a panel of experts (Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC)) did not recommend the MCF-7 cell proliferation assay (E-Screen) as part of US EPA's EDSP Tier 1 Screening battery (www.epa.gov/endo).

Potentially more relevant and interesting studies have examined the *in vitro* transforming effects of BBP on MCF-10F cells using a collagen assay (Fernandez and Russo, 2010; Huang *et al.*, 2007). In this assay, MCF-10F cells are induced to form duct-like structures and solid masses in response to estrogenic substances. Although BBP at high concentrations (i.e., 1 and 10 uM) induced modest increases in MCF-10F cell invasion capacity relative to controls, it did not induce the formation of duct-like or solid mass structures. In contrast, another weak estrogenic endocrine disruptor, Bisphenol A (BPA), did induce duct-like structures and the formation of solid masses (Fernandez and Russo, 2010). The authors concluded that only "BPA as well as 17β-estradiol are able to induce the neoplastic tranformation of human breast epithelial cells" (Fernandez and Russo, 2010). To date, there have been no other peer reviewed publications using this assay. Additional studies investigating other estrogenic endocrine disruptors are required to evaluate the overall reliability of this assay.

Diet and environmental factors are potential contributors to breast cancer risk. More recently, attention has focused on changes in the hormonal environment during critical stages of development that may modify the architecture and biological characteristics of the developing mammary gland, increasing its future susceptibility to cancer. Two cited studies examining morphological and gene expression changes in the rat mammary gland following developmental exposure (post-natal days 2-20 and day 10 post-conception to delivery) exposure to 500 mg/kg/d BBP provide compelling data that BBP does not contribute to mammary carcinogenesis (Moral *et al.*, 2011; Moral *et al.*, 2007). They report that "BBP did not induce significant changes in the morphology of the gland, but changed the proliferation index" of several mammary gland structures (i.e., terminal end bud (TEB) at 35 days but not at 21, 50 and 100 days and lobule type 1 structures (Lob1) at 35, 50 and 100 days) in the post-natal study (Moral, et al., 2007). They further describe the TEB changes as "subtle" and "slight" at doses that far exceed human exposures. The *in utero* study (day 10 post-conception to delivery) reports trends in changes in epithelial structures (e.g., terminal end buds, terminal ducts, alveolar buds, lobules type 1) that are not statistically significant, and increases in the proliferation index that are modest, transient and only statistically significant at select times (TEB only at 35 days, terminal ducts only at 100 days, Lob1 only at 100 days). Although increases in the proliferation index suggest these structures are more susceptible to carcinogenesis, Singletary et at., report that 500 mg/kg BBP significantly reduced *in vivo* formation of mammary DNA adducts by 95%, and mammary adenocarcinomas by 70% induced by 1,12-dimethylbenz[a]anthracene (DMBA) (Singletary *et al.*, 1997) .

Mammary gland differential gene expression is reported in both studies with functions associated with proliferation, differentiation, immune function, cell signaling and metabolism. However, these changes are also modest, and not anchored to a phenotypic response (Boverhof and Zacharewski, 2006; Paules, 2003; Waters *et al.*, 2008). More specifically, these studies did not confirm that changes in gene

expression resulted in a phenotypic or apical response such as an increase in protein expression or enzyme activity. Exposure to any substance at the appropriate dose will cause changes in gene expression, but this may not lead to changes in protein expression, enzyme activity or an adverse affect. For example, despite BBP induced changes in mammary gland gene expression, there is no reported evidence of BBP causing mammary carcinogenesis in high dose multigenerational reproductive and developmental toxicity studies (Kamrin, 2009; Kavlock *et al.*, 2002; Tyl *et al.*, 2004).

Conclusion

BBP is an excellent example of a chemical that exhibits very weak *in vitro* estrogenic activity but does not induce significant estrogenic activity *in vivo* (Ashby *et al.*, 1997a; Moore, 2000). Although *in vitro* assay results suggest effects on MCF-7 cell proliferation (E-Screen), estrogen receptor mRNA expression, 17β-estradiol-induced MCF-7 apoptosis, and recombinant yeast reporter gene activity, these responses are not predictive of *in vivo* responses. Furthermore, the modest changes in mammary gland proliferation and gene expression did not result in statistically significant morphological or phenotypic effects, consistent with the lack of mammary gland carcinogenesis in multigenerational reproductive and developmental toxicity studies at doses that far exceed human exposures. In conclusion, the weight of evidence indicates that BBP is not carcinogenic via an estrogenic mode of action.

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